

Effective Photodynamic therapy with Ir(III) for virulent clinical isolates of Extended-Spectrum beta-lactamase *Klebsiella pneumoniae*

Running title: aPDI for MDR virulent *K. pneumoniae*

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Abstract: *Background:* The extended-spectrum beta-lactamase (ESBL) *Klebsiella pneumoniae* is one of the leading causes of health-associated infections (HAI), whose antibiotic treatments have been severely reduced. Besides, HAI bacteria may harbor pathogenic factors such as siderophores, enzymes, or capsules, which increase the virulence of these strains. Thus, new therapies such as antimicrobial photodynamic inactivation (aPDI) are needed.

Method: A collection of 118 clinical isolates of *K. pneumoniae* were characterized susceptibility and virulence through the determination of the minimum inhibitory concentration (MIC) of Amk, Cfx, Cfz, Imp, Mer, and Pip-taz, and by PCR, the frequency of the virulence genes, K2, *magA*, *rmpA*, *entB*, *ybtS*, and *allS*. Susceptibility to innate immunity, such as human serum, macrophages, and polymorphonuclear cells, was tested. All the strains were tested for sensitivity to the photosensitizer PSIR-3 (4µg/mL) in a 17µW/cm² for 30 min aPDI.

Results: A significantly higher frequency of virulence genes in ESBL than non-ESBL bacteria were observed. The isolates of the genotype K2⁺, *ybtS*⁺, and *allS*⁺ display enhanced virulence since they showed higher resistance to human serum as well as to phagocytosis. All strains are susceptible to the aPDI with PSIR-3 decreasing viability in 3log₁₀. The combined treatment with Cfx improved the aPDI to 6log₁₀ for the ESBL strains. The combined treatment is synergistic as it showed an FIC index value of 0.15.

Conclusions: The aPDT effectively inhibits clinical isolates of *K. pneumoniae*, including the more risky strains of ESBL-producing bacteria and the K2⁺, *ybtS*⁺, and *allS*⁺ genotype. The aPDI with PSIR-3 is synergistic with Cfx.

Keywords: antibiotic resistance, virulence factors, *Klebsiella pneumoniae*, Photodynamic therapy

1. Introduction

Klebsiella pneumoniae is one of the major health-associated infection (HAIs) producers worldwide, including pneumonia, urinary tract, and bloodstream infections [1, 2]. Besides, HAI-producing *K. pneumoniae* strains progressively accumulate more multiple-drugs resistance (MDR), including extended-spectrum β -lactamases (ESBL) and carbapenemases such as KPC [3-5]. Treatment options for infections caused by MDR strains of *K. pneumoniae* are severely reduced to colistin and tigecycline [6-8]. The high MDR shown by strains of *K. pneumoniae* does not fully explain its notable success as one of the most important agents of HAIs, suggesting the participation of other factors [9]. An increasing number of authors suggest that increased bacterial survival during infections is related to virulence factors [1, 3, 9-14].

Virulence factors shown by *Klebsiella pneumoniae* strains are common to enterobacteria, such as siderophores, enzymes, and capsules [9]. These factors may be part of genes conserved amongst all *K. pneumoniae*, called the core set of genes, but other genes vary in frequency between strains, and they are part of the accessory set of genes [9]. During colonization and infection, *K. pneumoniae* requires the expression of several core and accessory genes to deal with, for example, the nutritional stress due to nutrient sequestration or the immune response of the host [15]. For instance, the acquisition of iron is facilitated during lung infection by the siderophores enterobactin (*entB*) and yersiniabactin (*ybtS*) genes encoded on the bacterial chromosome [1, 16]. Like iron, during infections of the lungs, urinary tract, and bloodstream, access to nitrogen is limited. The *allS* gene allows *K. pneumoniae* to use allantoin degradation as an alternative nitrogen source [17, 18]. The polysaccharide capsule is involved, among others, in resistance to death by complement-mediated opsonophagocytosis [9], and inhibition of macrophages [19, 20]. Some capsular varieties (such as K2) may produce hypervirulent strains with characteristic phenotypes such as the hypermucoviscous [19]. Some hypervirulent phenotypes are associated with the expression of the mucoviscosity-associated gene A (*magA*) and the regulator of the mucoid phenotype A gene (*rmpA*) [21]. Also, the *rmpA* gene increases the ability of ESBL-producing strains to resist the bactericidal activity of serum and phagocytosis [22]. Since the virulence factors increase bacterial survival and may influence antibiotic resistance expression, the composition of the pool of the virulence gene of *K. pneumoniae* strains may be associated with the antibiotic susceptibility pattern [9, 13].

Due to the emergence of multi-drug resistance (MDR), the deficit of new antibiotics is one of the most pressing threats to human health in the 21st century [23]. Given the high risk to public health caused by the deficiency of new antibiotics, the use of complementary antimicrobial therapies non-antibiotic-based, such as antibacterial photodynamic inactivation (aPDI), emerges as a promising alternative [24]. The aPDI may support the lack of antibiotic therapies against MDR and KPC strains [24, 25]. The aPDI is a procedure based on the use of photosensitizer (PS) compounds to produce light-activated local cytotoxicity (photooxidative-stress) [26]. The PSs work by energy absorb of a specific wavelength in the UV-Vis range, which is then transferred to molecular oxygen in solution to produce reactive oxygen species (ROS) [27]. Molecular O₂ can accept this energy together with electrons, or it can only undergo a one-electron reduction to produce superoxide anion radical (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radical (HO[•]) [28, 29]. The energy transferred to the O₂ without one-electron reduction produces singlet oxygen (¹O₂) [27]. The ROS production generates photooxidative-stress induced by aPDI, which occurs mainly due to the action of ¹O₂. The oxidative action of ¹O₂ occurs on organic molecules close to the PS through concerted addition reactions of alkene groups [30]. These organic molecules can be structural proteins or lipids of the bacterial envelope; therefore, the damage occurs to the cell wall plasma membrane or other bacterial structures, leading to nonspecific cell death [27, 31].

Previously, a PS compound based on a polypyridine Ir(III) complex (PSIR-3, see **Figure 1B**) demonstrated aPDI activity, inhibiting the bacterial growth of CKP and synergism with imipenem [32, 33]. In this work, we determine the frequency of the *K2*, *entB*, *ybtS*, *allS*, *rmpA*, and *magA* virulence genes, in a population of 118 clinical isolates of *K. pneumoniae* and evaluate their association to the susceptibility pattern to several antibiotics. Our data show virulence factors $K2^+$, $ybtS^+$, and $allS^+$ were associated with a modification in the bacterial minimum inhibitory concentrations (MICs) and increased ESBL production probability. These virulence genes significantly increased the survival of the bacteria, to innate immunity. These more virulent ESBL bacteria were all susceptible to the aPDI treatment with PSIR-3 and demonstrate synergism with cephalexime.

2. Materials and methods

2.1. Bacterial isolates

The ethics committee of the Faculty of Health Sciences, Central University of Chile, and the Central Metropolitan Health Service of Chile (MHSC) approved the study protocol and the informed consent form. The clinical isolates of *K. pneumoniae* were obtained from 122 samples from unrelated patients, received in the bacteriology laboratory of “Hospital el Carmen” (HEC) for a period of six months (2017 - 2018). The HEC is a complex hospital, with 412 beds and serving a population of more than 600,000 inhabitants. All clinical isolates were identified as *K. pneumoniae* following the protocols of the Institute of Clinical and Laboratory Standards (CLSI) [34]. As controls, the virulent strain (ATCC 43816 KPPR1) of susceptible *K. pneumoniae* ($K2^+$, $ybtS^+$, and $allS^+$) [35] and the MDR KPC⁺ ST258 (KP35) ($K2^-$, $ybtS^-$ and $allS^-$) [36] strains were also included.

2.2. Antimicrobial susceptibility testing

The MIC of the antimicrobial agents were determined in 96-well plates by micro-dilution methodology in cations-adjusted Muller-Hinton (ca-MHB) broth. Inoculum of 1×10^6 colony forming units (CFU) / mL of each clinical isolate was mixed with decreasing concentrations of each antibiotic and incubated overnight at 37 °C following the CLSI recommendations. The MIC for each antibiotic was determined as the last dilution in which no bacterial growth occurred, and the susceptibility intervals were assigned based on the cut-off points established by the CLSI (2018) for amikacin (Amk), cefotaxime (Cfx), ceftazidime (Cfz), imipenem (Imp), meropenem (Mer) and piperacillin-tazobactam (Pip-taz). According to the CLSI protocols [34], clinical isolates were strains considered ESBL-producing when resistant to cefotaxime but susceptible to the combination of Cfx / clavulanic acid. Values are presented as the median in mg/L and interquartile range (IQR).

2.3. DNA extraction and PCR amplification

The total DNA was obtained from stationary bacterial cultures in LB broth using the phenol-chloroform methodology. In brief, pelleted bacteria were suspended in 300 µL of 1x PBS and mixed with 300 µL of phenol: chloroform (25:24 vol: vol); the final mix was stirred well in a vortex and centrifuged at 13,000 x g at 4 °C for 15 minutes. The aqueous phase was mixed 1:1 with chloroform and centrifuged as above. Genomic and plasmidial DNA contained in the aqueous phase was precipitated in 0.6 vol of 2-propanol and sedimented at 13,000 x g at 4 °C for 20 minutes. The nucleic acids were washed twice with 70% ethanol and resuspended in 1 x Tris-EDTA buffer. PCR reactions were performed using 0.5 µM of each specific primer pair listed in **Table 1**, in 1x master mix GoTaq (Promega). The amplification was carried out at a final volume of 20 µL in a Veriti (Applied Biosystem) PCR machine with an initial denaturation step of 10 seconds at 95 °C, followed by 35 cycles of 10 sec at 95°C, 15 sec at 58 °C, and 30 seconds of

extension at 72 °C. A final extension step of 7 min at 72 °C was included, and PCR products were visualized on a 1.7% agarose gel.

Table 1. Primers for genes encoding virulence factors of *Klebsiella pneumoniae*

Gene	Primers	Gene Type	Amplicon size
<i>ybtS</i>	GACGGAAACAGCACGGTAAA GAGCATAATAAGGCGAAAGA	Siderophores	242
<i>entB</i>	GTCAACTGGGCCTTTGAGCCGTC TATGGGCGTAAACGCCGGTGAT	Siderophores	400
<i>magA</i>	GGTGCTCTTACATCATTGC GCAATGGCCATTTGCGTTAG	Capsular serotype K1 and hypermucoviscosity phenotype	128
<i>rmpA</i>	CATAAGAGTATTGGTTGACAG CTTGCATGAGCCATCTTTCA	Regulator of mucoid phenotype A	461
K2	CAACCATGGTGGTCGATTAG TGGTAGCCATATCCCTTTGG	Capsular serotype K2 and hypermucoviscosity phenotype	531
<i>allS</i>	CATTACGCACCTTTGTCAGC GAATGTGTCGGCGATCAGCTT	Allantoin metabolism	764
16S	ATTTGAAGAGGTTGCAAACGAT TTCACCTCTGAAGTTTCTTGTGTTC	Gene encoding the 16S ribosomal RNA	133

2.4. *K. pneumoniae* survival to innate immunity

To evaluate the survival of *K. pneumoniae* to the innate immunity, the bactericidal activity of human serum (NHS), as well as phagocytosis by human macrophages (MΦ), and polymorphonuclear cells (PMN) was determined. Both serum and leukocytes were obtained from blood samples of healthy voluntary donors who had not taken any antibiotic or anti-inflammatory medication for at least ten days before the day of sampling.

2.4.1. Susceptibility to normal human serum.

Serum susceptibility was carried out as before [37]; in brief, 75 μL of pooled NHS were mixed with 25 μL suspension containing 2 x 10⁶ CFU of each isolate in a 96-well plate. As a control, bacterial cultures of each isolate were mixed with 1x PBS. The mixtures were incubated at 37 °C for 3 h, and viable bacteria were enumerated by serial micro-dilution and colony counting on ca-MH agar plates. Serum resistance is expressed as viable bacteria in CFU / mL compared to untreated isolates controls.

2.4.2. Susceptibility to phagocytosis by macrophages and polymorphonuclear cells

The separation of mononuclear and polymorphonuclear cells was performed by centrifugation in a gradient density column of Histopaque (Sigma-Aldrich), following the manufacturer instructions. Monocytes were selected from other mononuclear cells by incubation in RPMI-1640 medium without fetal bovine serum (FBS) and differentiated into macrophages incubating during 7-9 days in RPMI-1640 10% FBS at 37 °C with 5% CO₂ [38]. The macrophage monolayer was infected with 2.5 x 10⁷ CFU (MOI 50:1) of each bacterial isolate and centrifuged at 200 x g for 5 minutes to synchronize phagocytosis. After 2 h of incubation, cells were washed and incubated for an additional 60 minutes in RPMI-1640 + 100 μg/mL gentamicin. Macrophages were lysed with 0.1% saponin for 10 minutes at room temperature, and

viable bacteria were enumerated as above. For PMN assays [39], 5×10^5 cells were combined with 5×10^6 CFU of each isolate (MOI 10:1) in serum-free RPMI-1640 and synchronize by centrifugation at $524 \times g$ for 8 minutes at 4°C . After 3h, PMNs were lysed with 0.1% saponin, and viable bacteria were enumerated. Control groups of non-phagocytosed bacteria were included.

2.5. Synthesis of the PSIR-3 compound

The structural and photophysical characterization of the PSIR-3 compound was described previously [40]. The complex synthesized can be described using the following general formula: $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})](\text{PF}_6)$, where $\text{N}^{\wedge}\text{N}$ is the ancillary ligand; and $\text{C}^{\wedge}\text{N}$ corresponds to a cyclometalating ligand. In this study PSIR-3 is $[\text{Ir}(\text{ppy})_2(\text{ppdh})]\text{PF}_6$ where ppdh is pteridino[7,6-f][1,10]phenanthroline-1,13(10H,12H)-dihydroxy and ppy is 2-phenylpyridine [41]. The structure and purities of the compound were confirmed by nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FT-IR), and mass spectroscopy (MALDI-MS) measurements. The absorption spectra were measured in acetonitrile (ACN) solutions using a Shimadzu UV-Vis Spectrophotometer UV-1900. The molar extinction coefficients of the characteristic bands were determined from the absorption spectra. Photoluminescence spectra were taken on an Edinburgh Instrument spectrofluorimeter using ACN solutions of the compounds previously degassed with N_2 for approximately 20 min. The emission quantum yields (Φ_{em}) were calculated according to the description of the literature [42]. Fluorescence lifetimes were measured using a time-correlated single-photon counting (TC-SPC) apparatus (PicoQuant PicoHarp 300) equipped with a subnanosecond LED source (excitation at 380 nm) powered by a PicoQuant PDL 800-B variable (2.5–40 MHz) pulsed power supply.

2.6. Antimicrobial activity of photosensitizer compounds

Stock solutions of 2 g/L of the PSIR-3 compound solubilized in ACN were used to prepare working solutions in distilled water. For the antimicrobial assay, the collection of 118 clinical isolates of *K. pneumoniae* was used, and the control strains of susceptible *K. pneumoniae* KPPR1 and the MDR strain ST258 were also included. All bacteria were grown as axenic culture in Luria Bertani broth or agar medium as appropriate. PSIR-3 was mixed in 24-well plates at a final concentration of 4 mg/L for photodynamic experiments, with suspensions of 1×10^7 colony forming units (CFU)/mL of each bacterium, in a final volume of 500 μL of cation-adjusted Muller Hinton (ca-MH) broth. Exposure to light was performed for 30 min in a chamber with a white LED lamp at a photon flux of $17 \mu\text{W}/\text{cm}^2$. After exposure to light, the CFUs of the viable bacteria were determined by broth-micro dilution and sub-cultured on ca-MH agar plates. Following the recommendations of the Clinical & Laboratory Standards Institute (CLSI 2017) [34], the agar plates were incubated during 16-20 h at 37°C in the dark, and colony count was recorded using a stereoscopic microscope. Control wells with bacteria culture with no photosensitizer or photosensitizer but not exposed to light were also included.

2.7. Determination of the synergy between PSIR-3 and Cfx

The fractional inhibitory concentration index (FIC) value was determined using the following formula [43, 44]. MIC_{ac} is the MIC of a compound A, combined with a compound B, and MIC_{bc} is the MIC of the compound B combined with the compound A. The MIC_a and MIC_b are the MIC of the A and B compound alone, respectively. Values in the FIC index ≤ 0.5 will be considered synergistic, and values > 4 will be considered antagonistic [44].

$$\text{FIC Index} = \frac{\text{MIC}_{ac}}{\text{MIC}_a} + \frac{\text{MIC}_{bc}}{\text{MIC}_b}$$

To determine the MIC-Cfx combined with each PSs, 1×10^7 UFC/mL of ESBL-producing bacteria were aPDI treated for 30 min with 4 mg/L of each PS and mixed with serial dilution (32 – 0.125 mg/L) of Cfx in ca-MH broth as above. To determine the MIC-PSs combined with Cfx, 1×10^7 UFC/mL of ESBL-producing bacteria were mixed with serial dilution of each PSs (32 – 0.125 mg/L) and a fixed concentration of 4 mg/L of Cfx, and subjected to aPDI as above.

2.7. Statistical analyses

Statistical analyses were performed using the Systat 13.2 software (Systat Software, Inc) and GraphPad v6.01 (Prism) software. The X^2 test or Fisher's exact test for categorical variables and the Mann-Whitney U test for continuous non-parametric variables were used. The risk of virulence genes to modify the MIC of the antibiotic was established by determining the odds ratio with a CI: 95%.

3. Results

3.1. Demographic characterization

This work seeks to demonstrate the capacity of aPDI to inhibiting the growth of clinical isolates of *K. pneumoniae*, which are diverse in antimicrobial susceptibility, genotype, and virulence. Phenotypic and genotypic characterization was conducted to determine the frequency of genes encoding virulence factors, the MIC values determined for various antibiotics, and susceptibility to innate immune components. From the clinical isolates of *K. pneumoniae* received in the laboratory, 118 were selected from different unrelated patients. The isolates were recovered mainly from urine samples, 113 (95.8%), and only five from respiratory samples (three endotracheal aspirates (2.5%) and two expectorations (1.7%)). As shown in **Table 2**, the samples were obtained from 78 (66%) females and 40 (34%) males, from 12 clinical services, with a higher contribution from the emergency room 41 (34.75%), secondly the unit of medicine 24 (20.34%), third ambulatory 16 (13.56 %) and in the fourth place urology 14 (11.86%). Of the 118 clinical isolates, 114 came from adults and 4 from pediatric patients. As shown in **Figure 1A**, the patients age fluctuated between 7 months and 94 years with a median (IQR: 25-75%) of 69.5 (54.8 – 84) years for females, and between 10 month and 92 years with a median of 68.5 (60.3 – 80.8) years for males. There are no significant differences in age between genders ($p = 0.279$ Mann-Whitney U test). The results confirm the infections with MDR and not MDR *K. pneumoniae* mainly affects the elderly population. The elderly are the most susceptible population to present complications derived from infectious diseases [45].

Table 2: Frequency of *K. pneumoniae* isolation by hospital unit and genre

Service	Female	Male	Total	Percent
Outpatient	12	4	16	13,56%
surgery	5	5	10	8,47%
Endocrinology	1	0	1	0,85%
Geriatrics	1	2	3	2,54%
Gynecology	2	0	2	1,69%
Home hospitalization	1	0	1	0,85%
Medicine	2	0	24	20,34%
Medical-surgical service	13	11	2	1,69%
Pediatrics	1	0	1	0,85%

Critical patient unit	2	1	3	2,54%
Emergency room	31	10	41	34,75%
Urology	7	7	14	11,86%
TOTAL	78	40	118	100%

3.2. Antibiotic susceptibility

In the *K. pneumoniae* population, the median and IQR (25-75%) of the MIC were determined and expressed in mg/L in a log₂ box plot. As shown in **Figure 1B**, the bacterial population was mainly susceptible to Amk with a median (IQR) of 8 (4-20). Similar to Amk, the population was mainly susceptible to carbapenem, Imp, and Mer with a median of 1 (0.5 - 1) and 1 (0.5 - 2), respectively. In contrast, most of the population was resistant to the cephalosporins, Cfx, and Cfz, with medians of 8 (8-8) and 32 (32-32). Finally, similar to cephalosporin, almost the entire population was resistant to the Pip-Taz combination with a median of 256 (64-256). From the population, 66 isolates resistant to Cfx were susceptible to the combination Cfx / clavulanic acid. Those isolates were identified as ESBL-producing bacteria.

3.3. Virulence gene frequency

In this work, we select certain virulence genes belonging to families with different mechanisms of action. PCR determined the frequency of carrying genes encoding the virulence factors *rmpA*, *magA*, *K2*, *entB*, *allS*, and *ybtS* for each isolate using specific primers (**Table 1**). These genes were selected for being representatives of different families of virulence factors. As shown in **Figure 1C**, the harboring frequency was; *rmpA* [2.54% (3/118)], *magA* [0% (0/118)], *K2* [39% (46/118)], *entB* [83.9% (99)], *allS* [11.2% (13/118)] and *ybtS* [73.8% (87/118)]. The most frequent virulence factor was the *entB* gene, followed by the *ybtS* gene. There were no isolates with the *magA* gene, and only three isolates harbor the *rmpA* gene. No isolates showed the hypermucoviscosity phenotype (by string test) in agar plates in the population of 118 unrelated isolates.

3.4. Correlation of virulence factors with antibiotic resistance

As shown in **Figure 1D**, there is a significantly higher frequency of ESBL strains that harbor three or more virulence factors compared to non-ESBL strains (Fisher's; $p < 0.026$). The non-parametric Mann-Whitney *U* test (Systat 13 software) was used to determine the association that each virulence gene has on the median MIC value assuming a null hypothesis $\alpha = 5\%$. As shown in **Table 3**, the *rmpA* gene was not significantly associated with a modification of the median-MIC of any of the antibiotics analyzed in this study ($p > 0.05$). On the other hand, the *allS* gene significantly influenced the median-MIC of Cfx, Cfz, and Pip-Taz ($p < 0.05$). Similarly, the *K2* gene significantly influenced the median-MIC of Cfx and Pip-Taz ($p < 0.05$). The *entB* gene significantly affected the median-MIC of Amk and Imp ($p < 0.05$). The *ybtS* gene significantly influenced the median-MIC of Cfx ($p < 0.006$). Finally, the *allS* gene significantly influenced the median-MIC of Cfx and Cfz antibiotics ($p < 0.05$). The MIC of the antibiotics more sensitive to the presence of virulence factors were the Cfx (sensitive to *K2*, *ybtS*, and *allS* genes) and Pip-Taz (sensitive to the *K2* and *allS* genes). This data shows that regardless of the patient's conditions, the multi-virulence is effectively an independent risk factor that promotes ESBL-production of clinical populations of *K. pneumoniae* with a $p < 0.026$ Fisher's exact test.

Table 3. Mann–Whitney U test p -values for modification of antibiotic MIC by virulence genes.

	Amikacin	Cefotaxime	Ceftazidime	Imipenem	Meropenem	Pip-Tazo
<i>rmpA</i>	0.285	0.051	0.432	0.363	0.527	0.411
K2	0.158	0.002	0.919	0.102	0.588	0.001
<i>entB</i>	0.009	0.918	0.117	0.015	0.291	0.072
<i>ybtS</i>	0.572	0.006	0.722	0.340	0.502	0.067
<i>allS</i>	0.326	0.024	0.001	0.086	0.537	0.013

Values in bold represent a $p < 0.05$, below the null hypothesis value with $\alpha = 5\%$, means a significant difference in MIC due to the presence of the virulence factor.

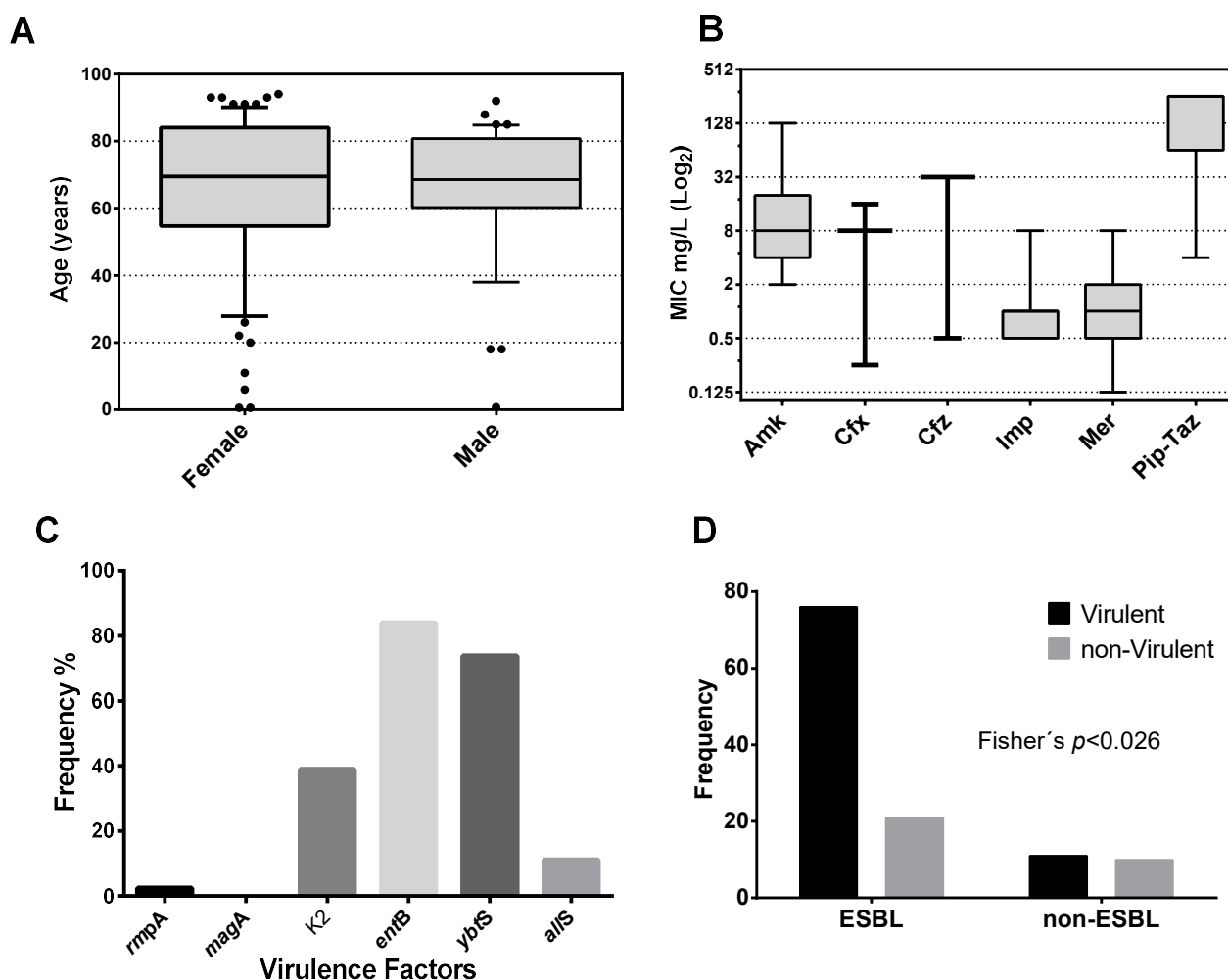


Figure 1. Characterization of patients and clinical isolates of *K. pneumoniae*. **A.** Box plot for the age of patients stratified by gender. Patients show a median of 69.5 years for females and 68.5 years for males. **B.** Box plot showing the median in the population of the minimum inhibitory concentration (MIC) of the seven antibiotics commonly used to treat infections by Gram-negative bacteria. **C.** Frequency of carrying of the six genes evaluated in the study population. **D.** Frequency distribution of carriers of three or more virulence factors stratified by ESBL production.

As shown in **Figure 2**, the box plot for each antibiotic stratified by the presence or absence of virulence genes was constructed to verify if the influence is to increase or decrease the median of the MIC. The presence of the *entB* gene is associated with a decrease in the median-MIC for Amk and Imp. On the other hand, the *K2* gene is associated with an increase in the median-MIC for Cfx and Pip-Tazo. Similarly, the *ybtS* gene is associated with an increase in the median-MIC for Cfx. Finally, the *allS* gene is associated with an increased median-MIC for Cfz and decreased median-MIC for Cfx and Pip-Tazo. Some of the virulence factors studied here were associated with the median- MIC modification for several antibiotics. In the *K. pneumoniae* population tested, the most influencing virulence factors were *entB*, *ybtS*, and *allS* genes. The stratified MICs-box plot made it possible to distinguish how virulence genes modulate antibiotic susceptibility by increasing or decreasing. Remarkably, the sum of the genes of the *K2*⁺, *ybtS*⁺, and *allS*⁺ genes contributes to increasing the median-MICs to values higher than the clinical susceptibility breakpoint established by the CLSI. These increased values occur similarly when efflux pumps are activated in other Gram-negative bacteria [46]. For example, in a population of *Pseudomonas aeruginosa*, the combined overexpression of the *mexA* and *mexX* efflux pump increased the median MIC for ciprofloxacin and cefepime above the cut-off points [46, 47].

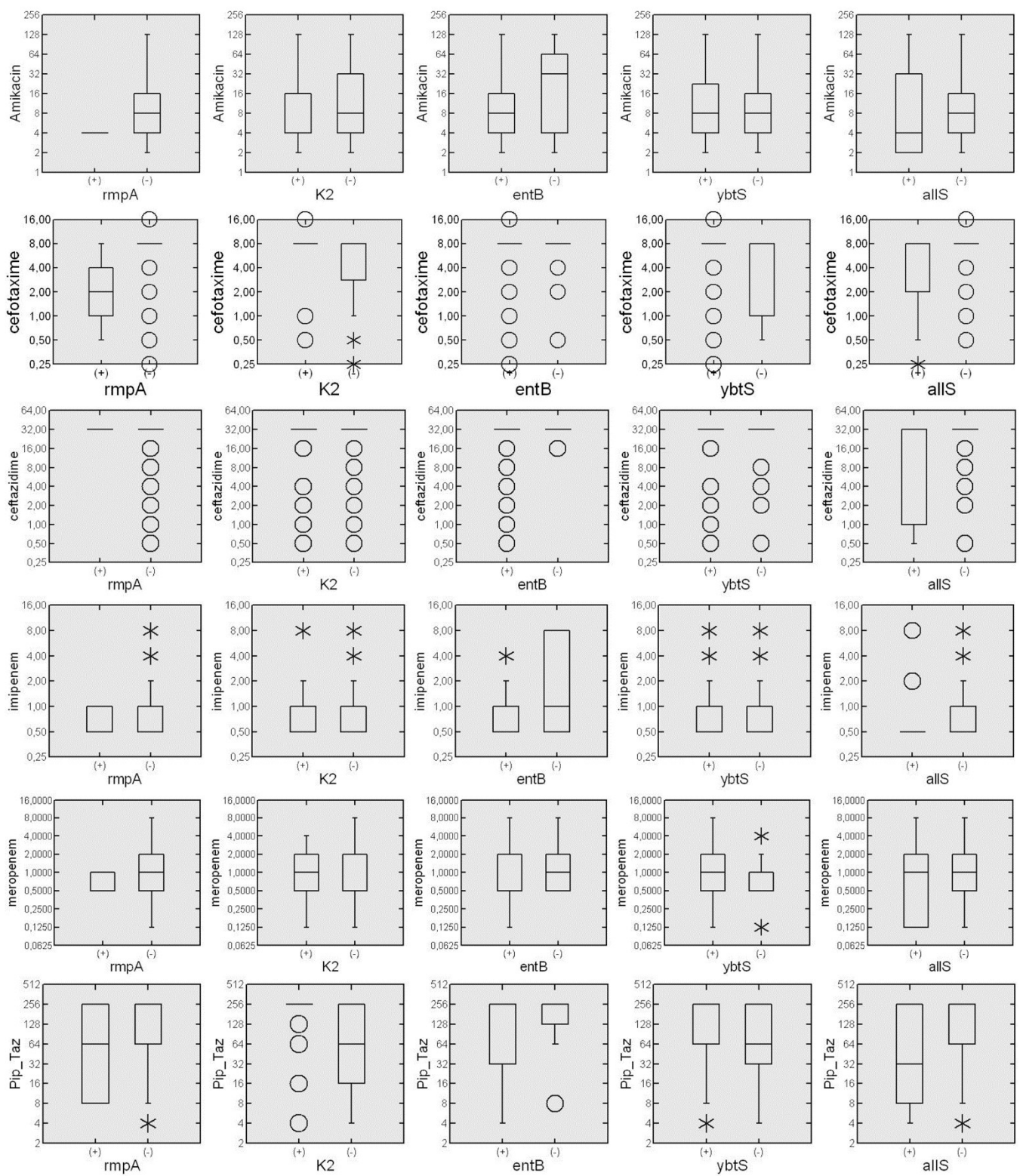


Figure 2. Association of virulence genes in the measurement of median MIC. The box diagrams created with the Systat 13 software summarize the MIC measures for six commonly used antibiotics,

stratified by the presence or absence of each of the six virulence genes. The data are presented as mg/L in a Log₂ scale of MICs for each antibiotic stratified by the presence (+) or absence (-) of each virulence gene. The horizontal lines represent the median, and the frequency limits between 25-75% of the individuals, the hollow dots represent single individuals, and the asterisks represent small groups of individuals.

3.5. Association of K2⁺, ybtS⁺ and allS⁺ virulence genes to survive the innate immunity

Our results show that the K2 and ybtS virulence genes are risk factors for the production of ESBL and that the allS gene acts as a protective factor. Then, we selected two groups of clinical isolates of *K. pneumoniae*, which harbor or not these three genes, to assess their susceptibility to innate immunity components. Only three isolates, identified by the numbers 81, 92, and 111, are of the K2⁺, ybtS⁺, and allS⁺ genotype, and unexpectedly all of them are ESBL-producers. From the genotype K2⁻, ybtS⁻ and allS⁻, the isolates 13, 21, and 22 were selected as being non-ESBL. As controls, the susceptible but highly virulent *K. pneumoniae* KPPR1 (K2⁺, ybtS⁺, and allS⁺) and the MDR but less virulent ST258 (K2⁻, ybtS⁻ and allS⁻) strains were also included.

To determine the bacterial susceptibility to normal human serum (NHS), they were exposed for 3 hours, and then the number of viable bacteria was determined by microdilution and plate counting. As shown in **Figure 3A**, the presence of all three virulence genes, K2⁺, ybtS⁺, and allS⁺, significantly (** = $p < 0.003$; two-way ANOVA) increases the survival of ESBL-producing bacteria compared to non-ESBL bacteria lacking all three virulence genes. On average, the serum resistance was improved by four orders of magnitude (4log₁₀). Similar to that observed with serum, ESBL-producing bacteria of the K2⁺, ybtS⁺, and allS⁺ genotype show a significant (**** = $p < 0.0001$) increase, 4log₁₀, in the survival to the MΦ activity, compared to non-ESBL bacteria that lacks these virulence genes (**Fig. 3B**). Finally, comparable to MΦ, survival to the activity of PMNs, of the bacteria of genotype K2⁺, ybtS⁺ and allS⁺, increased significantly (* = $p < 0.02$), at least one time on average, compared to non-ESBL strains that lack the virulence genes (**Fig. 3C**). As shown in **Figure 3**, the virulent control KPPR1 strain was more resistant to serum (**Fig. 3D**), macrophages (**Fig. 3E**), and PMN (**Fig. 3F**) compared to the MDR ST258 *K. pneumoniae* strain. Consistently the clinical control isolates have shown to be more susceptible to the bactericidal activity of the serum (**3D**) and the phagocytic activity of MΦ (**3E**) and PMN (**3F**). The co-occurrence of harboring multiple genes that encode virulence factors and the ESBL-production leads to enhanced virulence. The ESBL-producing strains of *K. pneumoniae* of the genotype K2⁺, ybtS⁺, and allS⁺ were more resistant to innate immunity, consistently with studies over MDR populations of *K. pneumoniae* that increased their 30-day mortality over patients undergoing bloodstream infections [1, 48]. Also, virulence genes, such as siderophores, which have an essential role in bacterial survival and virulence [49, 50], have been previously associated with the MDR-*K. pneumoniae* [16, 51]. In this study, ESBL-producer *K. pneumoniae* of the K2⁺, ybtS⁺ and allS⁺ genotype shown a survival improvement for killing by PMNs. Previously, the PMN has demonstrated a limited binding and uptake capacity for MDR-*K. pneumoniae* [39]. The activity of the PMN is the most important cellular component of the innate immune response, essential as the first line of defense against bacterial infections [52].

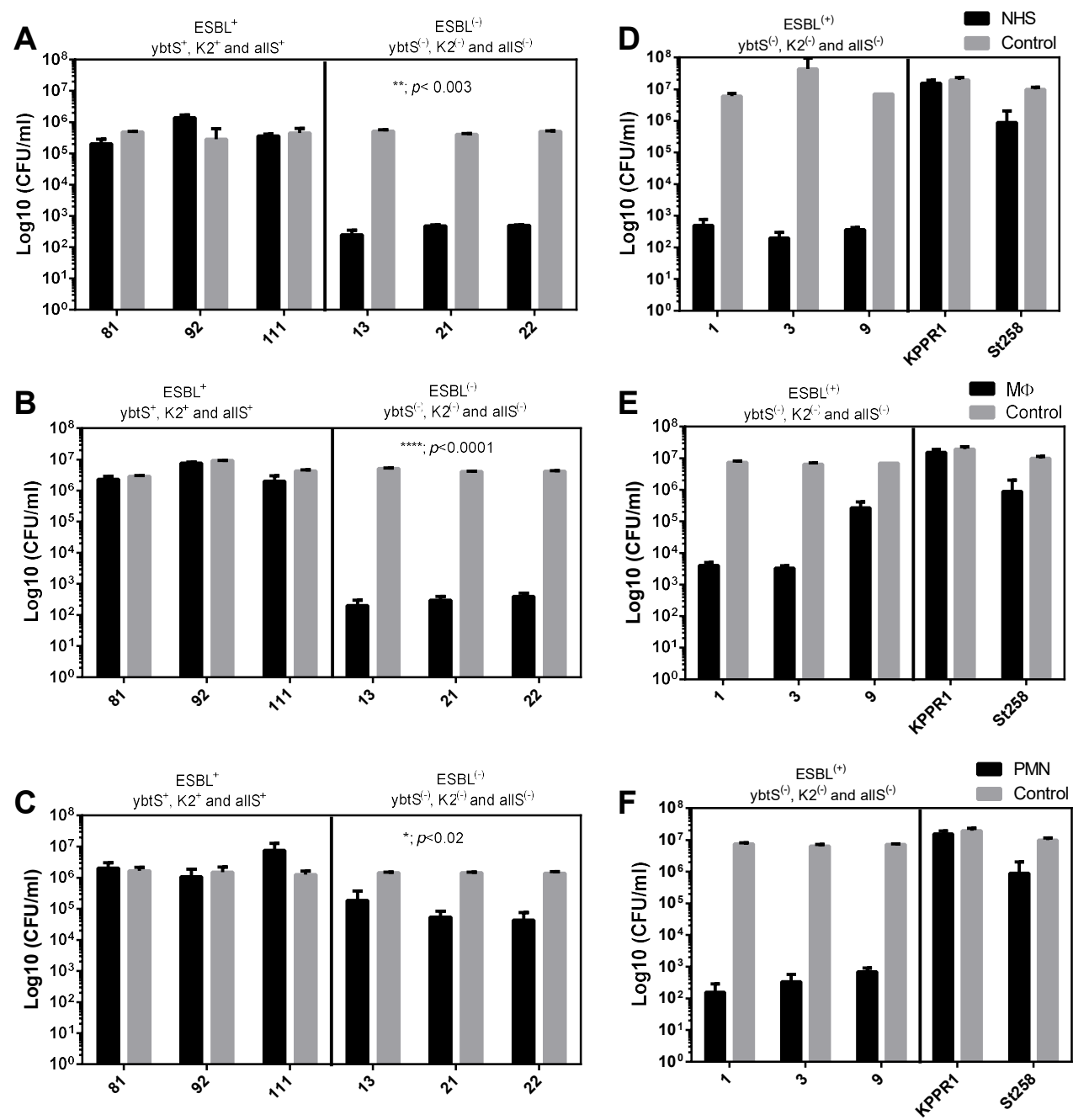


Figure 3. Effect of virulence genes on the susceptibility of ESBP or non-ESBP strains to components of innate immunity. The susceptibility of ESBP-producing *K. pneumoniae* clinical isolates bearing the virulence genes *ybtS*⁺, *K2*⁺, and *allS*⁺ was determined and compared with non-ESBP producing bacteria that lack the virulence genes. **A.** Susceptibility to normal human serum (NHS), **B.** Susceptibility to phagocytosis by macrophages (MΦ), and **C.** Susceptibility to phagocytosis by polymorphonuclear cells (PMN). The results of two independent experiments performed in triplicate are shown (n = 6). Viable bacteria were enumerated by colony count on ca-MH agar after serial micro-

dilution. The CFU / mL values are presented as means $\pm$ SD, on a $\log_{10}$ scale of treated bacteria (black bars) compared to untreated control bacteria (gray bars). **** = $p < 0.0001$, ** = $p < 0.003$ and * = $p < 0.02$ of two-way ANOVA comparing the proportion of treated / untreated ESBL bacteria with non-ESBL bacteria.

3.7. Susceptibility of clinical isolates to aPDI with PSIR-3

3.7.1. Photophysical properties of the PSIR-3 compound

We have previously shown that Ir(III)-based compounds such as PSIR-3 has photodynamic antimicrobial activity against imipenem-resistant *Klebsiella pneumoniae* [32, 33]. In this work, we tested a coordination compound characterized by a positive charge in the first coordination sphere (**Figure 4B**). The photophysical evaluation of the PSIR-3 performed in acetonitrile solution [40], revealed absorption processes at 375 and 392 nm attributable at the first instance to charge-transfer transitions (**Figure 4C and D**) [32]. When the PSIR-3 compound was excited with a wavelength corresponding to the lowest charge-transfer absorption energy, 375 nm, it showed maximum emission at 598 nm (**Figure 4C and D**). **Figure 4C** shows the recorded lifetimes of excited states in 0.32 μ s and the calculated quantum yield (Φ_{em}) in 0.011 [40]. The aPDI activity of the PSIR-3 compound was compared with the positive PS control [Ru(bpy)₃](PF₆)₂ (bpy = 2,2'-bipyridine) called PS-Ru. According to the literature, the PS-Ru shows a charge-transfer absorption process at 450 nm with maximum emission at 600 nm (excited in 450 nm) in acetonitrile [42], with Φ_{em} of 0.095 [42], and a lifetime registered of its excited state of 0.855 μ s (**Figure 4C**) [53].

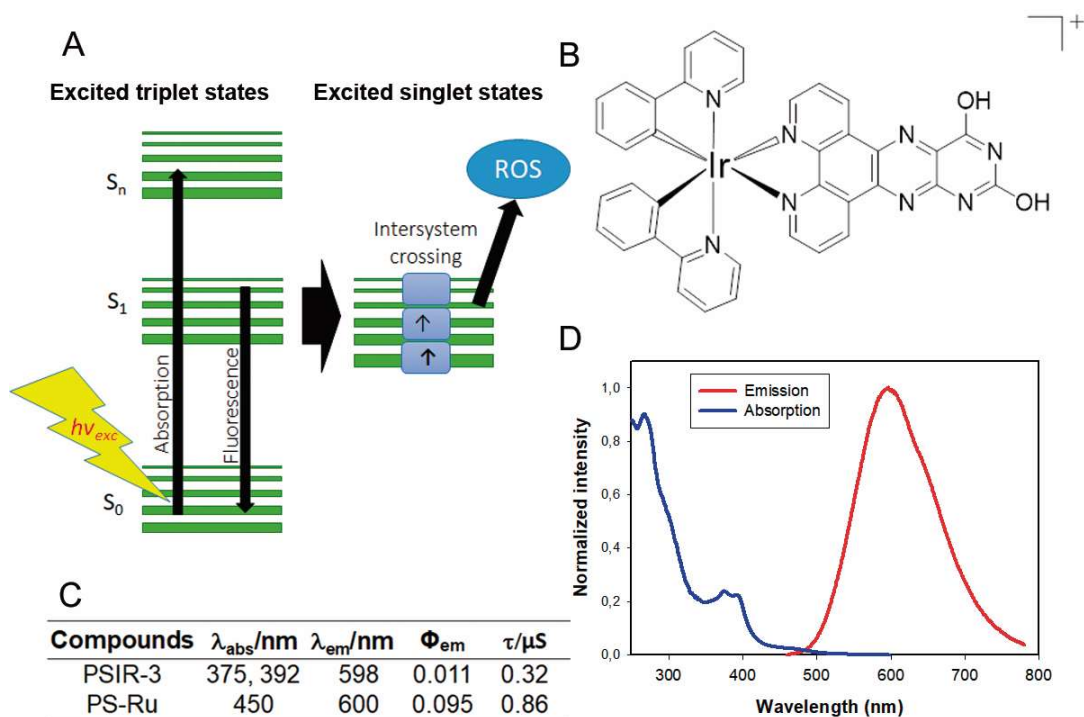


Figure 4. The Photofisical properties of the PSIR-3 photosensitizer. **A** schematic representation of the absorption-emission process of photosensitizer molecules. Light excites external electrons to accede from a basal state S_0 to a higher energetic state S_1-S_n . Suppose the electron return to its ground state, the energy is released as fluorescence. However, when the excited electron enters an intersystem crossing

process, the released energy excites molecular oxygen and converts it to ROS. **B** chemical structure of the Ir(III) compound (PSIR-3, [Ir(ppy)₂(ppdh)]PF₆). **C** Summary of the photophysical properties of the PSIR-3 and PS-Ru compounds. Where: λ_{abs} =wavelength of absorbance, λ_{em} = wavelength of emission, Φ_{em} = emission quantum yield, τ = time in the excited state. Data for PS-Ru was obtained from the literature [42, 53]. **D** The absorption and the emission spectra of the PSIR-3 in ACN.

3.7.2. Antimicrobial photodynamic inhibition of the PSIR-3 over clinical isolates

Photodynamic treatment was verified to produce the observed growth inhibition of the 118 clinical isolates of *K. pneumoniae* compared to the untreated bacteria. The photodynamic activity of the PSIR-3 compound was compared to the activity of the PS-Ru reference compound as a positive control [54-57]. As seen in **Figure 5**, compared to the control of untreated bacteria, photodynamic treatment with 4 $\mu\text{g/mL}$ PSIR-3 inhibits bacterial growth $> 3 \log_{10}$ ($> 99.9\%$) of both *K. pneumoniae* strains (**** $p < 0.0001$; compared to untreated control). The results show that the bactericidal effect produced by PSIR-3 is light-dependent ($ns = p > 0.05$; compared to the untreated control). These results are comparable with those obtained with the positive control compound PS-Ru, which has shown that bacterial growth inhibition is light-dependent (**** $p < 0.0001$; compared to the untreated control).

3.7.3. Synergism between aPDI with PSIR-3 and cefotaxime.

Since PSIR-3 showed synergism combined with imipenem [32], we analyzed whether it shows synergism with cefotaxime in the population of clinical isolates. First, it was determined whether the combined treatment with Cfx increases the inhibition of bacterial growth of aPDI with PSIR-3. The 118 clinical isolates of *K. pneumoniae* were exposed to the preparation of 4 $\mu\text{g/mL}$ of cefotaxime with 4 $\mu\text{g/mL}$ of PSIR-3 (its MIC). Control bacteria without Cfx and exposure to light were included. As expected, the PSIR-3 compound mixed with cefotaxime significantly (**** $p < 0.0001$) increased the bactericidal effect on the clinical isolates population from 3 to 6 $\log_{10}$ reduction (**Figure 5A**). As seen before, a significantly increased inhibitory effect was not observed when combining the cefotaxime with the PS-Ru control compound ($ns \ p > 0.05$). Secondly, the set of 66 clinical isolates characterized as ESBL-producing *K. pneumoniae* were treated with PSIR-3 aPDT for 1 h, and serial dilutions determined the MIC for Cfx in ca-MH broth. As seen in **Figure 5B**, a significant reduction (**** $p < 0.0001$) from 8 $\mu\text{g} / \text{mL}$ (8 -8) to 0.17 (0.17 – 0.333) on Cfx-MIC was observed compared to the untreated group. The combined treatment also reduced the PSIR-3-MIC, from 4 mg/L to 0.5 mg/L (**Figure 5C**). Similar to previously shown with imipenem [32], compound PSIR-3 produced a significant change in Cfx-susceptibility with a fractional inhibitory concentration (FIC) index of 0.15 (**Table 4**). **Figure 5C** shows the control compound, PS-Ru, did not significantly change Cfx-susceptibility with an FIC Index 1.58 (**Table 4**). Because synergy is defined as an FIC index of ≤ 0.5 [44], the increased inhibitory effect observed when combining PSIR-3 with Cfx is not summative but synergistic.

Table 4. FIC index calculation

Compounds	MIC	MICcombined	FIC	FIC Index
Cfx	8.00			
PSIR-3	4.00	0.17	0.02	0.15
		0.50	0.13	
PS-Ru	8.00	6.67	0.83	1.58
		6.00	0.75	

MIC values are de median for the ESBL-producing *K. pneumoniae*, n=66.

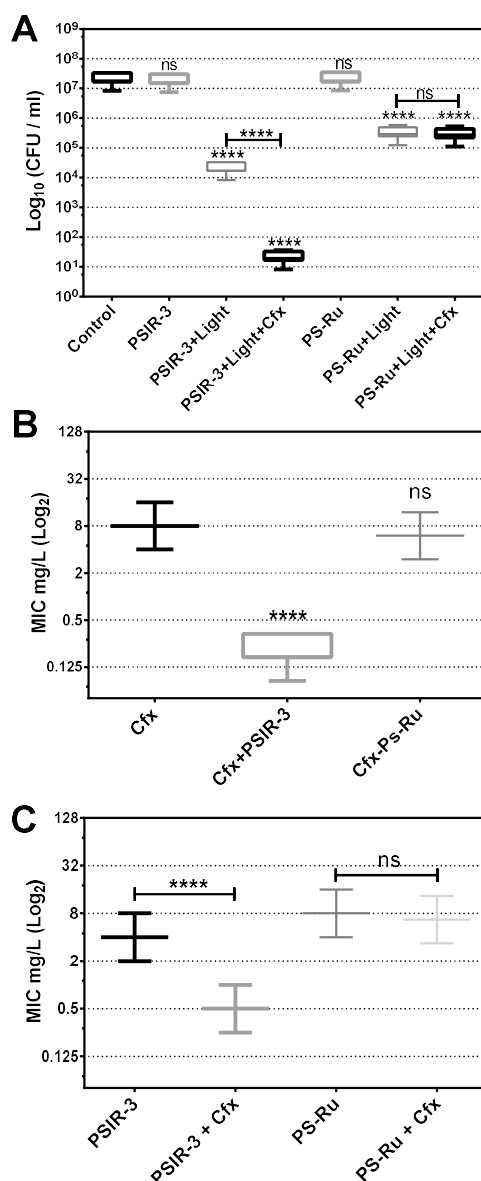


Figure 5 Antimicrobial photodynamic inactivation of clinical isolates of *K. pneumoniae*. **A.** Growth inhibition of 118 clinical isolates of *K. pneumoniae* subjected to aPDI with PSIR-3. The bacteria were used at a concentration of 1×10^7 CFU/mL and mixed in triplicate with 4 mg/L of PSIR-3 or PSIR-4 compounds. For the aPDI, the mixture of bacteria with PS was exposed for 1 hour at $17 \mu\text{W}/\text{cm}^2$ of white light. As a control, bacteria combined with the PSs not exposed to light (PSIR-3 or PS-Ru) and bacteria not combined with the PSs (control) were included. Colony count enumerated of viable bacteria on ca-MH agar after serial micro-dilution. The CFU/mL values are presented as means $\pm$ SD on a log₁₀ scale. **B.** From the clinical isolates, 66 ESBL-producing bacteria were exposed to aPDI using PSIR-3 or PS-Ru, and MIC for cefotaxime (Cfx) was performed in triplicate in ca-MH broth for 16 – 20 h. **C** For ESBL-producing bacteria, the MIC for PSIR-3 or PS-Ru, were determined in combination with 4 mg / L of Cfx, performed in triplicate in ca-MH agar for 16 – 20 h. The MIC values are presented as median $\pm$ SD of mg/mL on a log₂ scale. Not significant [ns] $p > 0.05$ by Student's *t*-test among treated bacteria compared to control; **** $p < 0.0001$ by Student's *t*-test among treated bacteria compared to control.

The behavior exhibited by PSIR-3 must be related, as mentioned in previous reports [32, 33], to the external substituents bonded to polypyridine ligand structures and affinity to bacterial envelope [58]. This synergism is also comparable to other photosensitizers; for example, rose bengal showed an increase in the susceptibility of *Acinetobacter baumannii* for a range of antibiotics used along with aPDI [59]. In another example, conventional antibiotics and alternative compounds reported synergism in a murine model for ESKAPE pathogens using anti-biofilm peptides [60].

In this work, we saw that multidrug-resistance and virulence are significant factors in clinical isolates of *K. pneumoniae*. However, the increase in MICs can be neutralized by aPDI, turning resistant strains susceptible. APDI is effective in treating multidrug-resistant bacteria and more virulent strains, as well as strains that combine both characteristics. The aPDI then becomes a great support to antimicrobial therapy in a shortage of new effective antibiotics.

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Compliance with Ethical Standards

Conflict of Interest: The authors declare no conflict of interest.

Ethical Approval: The study protocol and the informed consent form have been approved by The ethics committee of the Faculty of Health Sciences, of the Central University of Chile, and by the Bioethics Committee of the Central Metropolitan Health Service of Chile (MHSC), Act number: N° 124/07.

The study protocol and the informed consent form have been approved by Act number: N° 124/07.

References

- [1] D. Kim, B.Y. Park, M.H. Choi, E.J. Yoon, H. Lee, K.J. Lee, Y.S. Park, J.H. Shin, Y. Uh, K.S. Shin, J.H. Shin, Y.A. Kim, S.H. Jeong, Antimicrobial resistance and virulence factors of *Klebsiella pneumoniae* affecting 30 day mortality in patients with bloodstream infection, The Journal of antimicrobial chemotherapy 74(1) (2019) 190-199. doi: 10.1093/jac/dky397
- [2] X. Zhen, C.S. Lundborg, X. Sun, X. Hu, H. Dong, Economic burden of antibiotic resistance in ESKAPE organisms: a systematic review, Antimicrobial Resistance & Infection Control 8(1) (2019) 137. doi: 10.1186/s13756-019-0590-7
- [3] A.I. Lev, E.I. Astashkin, A.A. Kislichkina, E.V. Solovieva, T.I. Kombarova, O.V. Korobova, O.N. Ershova, I.A. Alexandrova, V.E. Malikov, A.G. Bogun, A.I. Borzilov, N.V. Volozhantsev, E.A. Svetoch, N.K. Fursova, Comparative analysis of *Klebsiella pneumoniae* strains isolated in 2012-2016 that differ by antibiotic resistance genes and virulence genes profiles, Pathogens and global health 112(3) (2018) 142-151. doi: 10.1080/20477724.2018.1460949
- [4] P. Tofas, A. Skiada, M. Angelopoulou, N. Sipsas, I. Pavlopoulou, S. Tsaousi, M. Pagoni, M. Kotsopoulou, S. Perloreztzou, A. Antoniadou, M. Pirounaki, A. Skoutelis, G.L. Daikos, Carbapenemase-producing *Klebsiella pneumoniae* bloodstream infections in neutropenic patients with haematological malignancies or aplastic anaemia: Analysis of 50 cases, International journal of antimicrobial agents 47(4) (2016) 335-9. doi: 10.1016/j.ijantimicag.2016.01.011
- [5] Y. Li, Q.L. Sun, Y. Shen, Y. Zhang, J.W. Yang, L.B. Shu, H.W. Zhou, Y. Wang, B. Wang, R. Zhang, S. Wang, Z. Shen, Rapid Increase in Prevalence of Carbapenem-Resistant Enterobacteriaceae (CRE) and Emergence of Colistin Resistance Gene mcr-1 in CRE in a Hospital in Henan, China, Journal of clinical microbiology 56(4) (2018) doi: 10.1128/JCM.01932-17
- [6] WHO, WHO publishes list of bacteria for which new antibiotics are urgently needed, (2017) doi:
- [7] C. Willyard, The drug-resistant bacteria that pose the greatest health threats, Nature 543(7643) (2017) 15. doi:
- [8] A.K. Pragasam, C. Shankar, B. Veeraraghavan, I. Biswas, L.E. Nabarro, F.Y. Inbanathan, B. George, S. Verghese, Molecular Mechanisms of Colistin Resistance in *Klebsiella pneumoniae* Causing Bacteremia from India-A First Report, Frontiers in microbiology 7 (2016) 2135. doi: 10.3389/fmicb.2016.02135
- [9] R.M. Martin, M.A. Bachman, Colonization, Infection, and the Accessory Genome of *Klebsiella pneumoniae*, Frontiers in cellular and infection microbiology 8 (2018) 4. doi: 10.3389/fcimb.2018.00004
- [10] T. Sonda, H. Kumburu, M. van Zwetselaar, M. Alifrangis, B.T. Mmbaga, O. Lund, G.S. Kibiki, F.M. Aarestrup, Molecular epidemiology of virulence and antimicrobial resistance determinants in *Klebsiella pneumoniae* from hospitalised patients in Kilimanjaro, Tanzania, European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology 37(10) (2018) 1901-1914. doi: 10.1007/s10096-018-3324-5
- [11] K.S. Khaertynov, V.A. Anokhin, A.A. Rizvanov, Y.N. Davidyuk, D.R. Semyenova, S.A. Lubin, N.N. Skvortsova, Virulence Factors and Antibiotic Resistance of *Klebsiella pneumoniae* Strains Isolated From Neonates With Sepsis, Frontiers in medicine 5 (2018) 225. doi: 10.3389/fmed.2018.00225
- [12] S. Rastegar, M. Moradi, D. Kalantar-Neyestanaki, D. Ali Golabi, H. Hosseini-Nave, Virulence Factors, Capsular Serotypes and Antimicrobial Resistance of Hypervirulent *Klebsiella pneumoniae* and Classical *Klebsiella pneumoniae* in Southeast Iran, Infection & chemotherapy (2019) doi: 10.3947/ic.2019.0027
- [13] S. Derakhshan, A. Hosseinzadeh, Resistant *Pseudomonas aeruginosa* carrying virulence genes in hospitalized patients with urinary tract infection from Sanandaj, west of Iran, Gene Reports (2020) 100675. doi: 10.1016/j.genrep.2020.100675
- [14] W. Imtiaz, Z. Syed, Z. Rafaque, S.C. Andrews, J.I. Dasti, Analysis of Antibiotic Resistance and Virulence Traits (Genetic and Phenotypic) in *Klebsiella pneumoniae* Clinical Isolates from Pakistan: Identification of Significant

Levels of Carbapenem and Colistin Resistance, *Infection and drug resistance* 14 (2021) 227-236. doi: 10.2147/IDR.S293290

[15] M.K. Paczosa, J. Mecsas, *Klebsiella pneumoniae*: Going on the Offense with a Strong Defense, *Microbiology and molecular biology reviews* : MMBR 80(3) (2016) 629-61. doi: 10.1128/MMBR.00078-15

[16] M.A. Bachman, J.E. Oyler, S.H. Burns, M. Caza, F. Lepine, C.M. Dozois, J.N. Weiser, *Klebsiella pneumoniae* yersiniabactin promotes respiratory tract infection through evasion of lipocalin 2, *Infection and immunity* 79(8) (2011) 3309-16. doi: 10.1128/IAI.05114-11

[17] Y.J. Kim, S.I. Kim, Y.R. Kim, S.H. Wie, H.K. Lee, S.Y. Kim, Y.J. Park, Virulence factors and clinical patterns of hypermucoviscous *Klebsiella pneumoniae* isolated from urine, *Infectious diseases* 49(3) (2017) 178-184. doi: 10.1080/23744235.2016.1244611

[18] J.B. French, D.B. Neau, S.E. Ealick, Characterization of the structure and function of *Klebsiella pneumoniae* allantoin racemase, *Journal of molecular biology* 410(3) (2011) 447-60. doi: 10.1016/j.jmb.2011.05.016

[19] K.A. Nadasy, R. Domiati-Saad, M.A. Tribble, Invasive *Klebsiella pneumoniae* syndrome in North America, *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 45(3) (2007) e25-8. doi: 10.1086/519424

[20] Y.P. Chuang, C.T. Fang, S.Y. Lai, S.C. Chang, J.T. Wang, Genetic determinants of capsular serotype K1 of *Klebsiella pneumoniae* causing primary pyogenic liver abscess, *The Journal of infectious diseases* 193(5) (2006) 645-54. doi: 10.1086/499968

[21] M. Cubero, I. Grau, F. Tubau, R. Pallares, M.A. Dominguez, J. Linares, C. Ardanuy, Hypervirulent *Klebsiella pneumoniae* clones causing bacteraemia in adults in a teaching hospital in Barcelona, Spain (2007-2013), *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 22(2) (2016) 154-60. doi: 10.1016/j.cmi.2015.09.025

[22] H.A. Lin, Y.L. Huang, K.M. Yeh, L.K. Siu, J.C. Lin, F.Y. Chang, Regulator of the mucoid phenotype A gene increases the virulent ability of extended-spectrum beta-lactamase-producing serotype non-K1/K2 *Klebsiella pneumoniae*, *Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi* 49(4) (2016) 494-501. doi: 10.1016/j.jmii.2014.08.023

[23] Y.Y. Liu, Y. Wang, T.R. Walsh, L.X. Yi, R. Zhang, J. Spencer, Y. Doi, G. Tian, B. Dong, X. Huang, L.F. Yu, D. Gu, H. Ren, X. Chen, L. Lv, D. He, H. Zhou, Z. Liang, J.H. Liu, J. Shen, Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study, *The Lancet. Infectious diseases* 16(2) (2016) 161-8. doi: 10.1016/S1473-3099(15)00424-7

[24] M. Valenzuela-Valderrama, I.A. Gonzalez, C.E. Palavecino, Photodynamic treatment for multidrug-resistant Gram-negative bacteria: perspectives for the treatment of *Klebsiella pneumoniae* infections, *Photodiagnosis and photodynamic therapy* 28 (2019) 256-264. doi: 10.1016/j.pdpdt.2019.08.012

[25] M.G. Tosato, P. Schilardi, M.F. Lorenzo de Mele, A.H. Thomas, C. Lorente, A. Minan, Synergistic effect of carboxypterin and methylene blue applied to antimicrobial photodynamic therapy against mature biofilm of *Klebsiella pneumoniae*, *Heliyon* 6(3) (2020) e03522. doi: 10.1016/j.heliyon.2020.e03522

[26] P. Agostinis, K. Berg, K.A. Cengel, T.H. Foster, A.W. Girotti, S.O. Gollnick, S.M. Hahn, M.R. Hamblin, A. Juzeniene, D. Kessel, M. Korbelik, J. Moan, P. Mroz, D. Nowis, J. Piette, B.C. Wilson, J. Golab, Photodynamic therapy of cancer: an update, *CA: a cancer journal for clinicians* 61(4) (2011) 250-81. doi: 10.3322/caac.20114

[27] K. Briviba, L.O. Klotz, H. Sies, Toxic and signaling effects of photochemically or chemically generated singlet oxygen in biological systems, *Biological chemistry* 378(11) (1997) 1259-65. doi: 9426185

[28] C.C. Winterbourn, A.J. Kettle, Redox reactions and microbial killing in the neutrophil phagosome, *Antioxidants & redox signaling* 18(6) (2013) 642-60. doi: 10.1089/ars.2012.4827

[29] L.A. Alvarez, L. Kovacic, J. Rodriguez, J.H. Gosemann, M. Kubica, G.G. Pircalabioru, F. Friedmacher, A. Cean, A. Ghise, M.B. Sarandan, P. Puri, S. Daff, E. Plettner, A. von Kriegsheim, B. Bourke, U.G. Knaus, NADPH oxidase-derived H₂O₂ subverts pathogen signaling by oxidative phosphotyrosine conversion to PB-DOPA, *Proceedings of the National Academy of Sciences of the United States of America* 113(37) (2016) 10406-11. doi: 10.1073/pnas.1605443113

- [30] E.C. Ziegelhoffer, T.J. Donohue, Bacterial responses to photo-oxidative stress, *Nature reviews. Microbiology* 7(12) (2009) 856-63. doi: 10.1038/nrmicro2237
- [31] A.C. Trindade, J.A. de Figueiredo, S.D. de Oliveira, V.C. Barth Junior, S.W. Gallo, C. Follmann, C.F. Wolle, L. Steier, R.D. Morgental, J.B. Weber, Histopathological, Microbiological, and Radiographic Analysis of Antimicrobial Photodynamic Therapy for the Treatment of Teeth with Apical Periodontitis: A Study in Rats' Molars, *Photomedicine and laser surgery* (2017) doi: 10.1089/pho.2016.4102
- [32] M. Valenzuela-Valderrama, N. Carrasco-Véliz, I.A. González, P. Dreyse, C.E. Palavecino, Synergistic effect of combined imipenem and photodynamic treatment with the cationic Ir(III) complexes to polypyridine ligand on carbapenem-resistant *Klebsiella pneumoniae*, *Photodiagnosis and Photodynamic Therapy*, **31** (2020) 101882. doi: 10.1016/j.pdpdt.2020.101882
- [33] V. Bustamante, I.A. Gonzalez, P. Dreyse, C.E. Palavecino, The mode of action of the PSIR-3 photosensitizer in the photodynamic inactivation of *Klebsiella pneumoniae* is by the production of type II ROS which activate RpoE-regulated extracytoplasmic factors, *Photodiagnosis and photodynamic therapy* 32 (2020) 102020. doi: 10.1016/j.pdpdt.2020.102020
- [34] CLSI, Performance Standards for Antimicrobial Susceptibility Testing, 27 ed.2017.
- [35] C.A. Broberg, W. Wu, J.D. Cavalcoli, V.L. Miller, M.A. Bachman, Complete Genome Sequence of *Klebsiella pneumoniae* Strain ATCC 43816 KPPR1, a Rifampin-Resistant Mutant Commonly Used in Animal, Genetic, and Molecular Biology Studies, *Genome announcements* 2(5) (2014) doi: 10.1128/genomeA.00924-14
- [36] D. Ahn, H. Penaloza, Z. Wang, M. Wickersham, D. Parker, P. Patel, A. Koller, E.I. Chen, S.M. Bueno, A.C. Uhlemann, A. Prince, Acquired resistance to innate immune clearance promotes *Klebsiella pneumoniae* ST258 pulmonary infection, *JCI insight* 1(17) (2016) e89704. doi: 10.1172/jci.insight.89704
- [37] H. Sahly, H. Aucken, V.J. Benedi, C. Forestier, V. Fussing, D.S. Hansen, I. Ofek, R. Podschun, D. Sirot, J.M. Tomas, D. Sandvang, U. Ullmann, Increased serum resistance in *Klebsiella pneumoniae* strains producing extended-spectrum beta-lactamases, *Antimicrobial agents and chemotherapy* 48(9) (2004) 3477-82. doi: 10.1128/AAC.48.9.3477-3482.2004
- [38] M.A. Ares, J.L. Fernandez-Vazquez, R. Rosales-Reyes, M.D. Jarillo-Quijada, K. von Bargen, J. Torres, J.A. Gonzalez-y-Merchand, M.D. Alcantar-Curiel, M.A. De la Cruz, H-NS Nucleoid Protein Controls Virulence Features of *Klebsiella pneumoniae* by Regulating the Expression of Type 3 Pili and the Capsule Polysaccharide, *Frontiers in cellular and infection microbiology* 6 (2016) 13. doi: 10.3389/fcimb.2016.00013
- [39] S.D. Kobayashi, A.R. Porter, D.W. Dorward, A.J. Brinkworth, L. Chen, B.N. Kreiswirth, F.R. DeLeo, Phagocytosis and Killing of Carbapenem-Resistant ST258 *Klebsiella pneumoniae* by Human Neutrophils, *The Journal of infectious diseases* 213(10) (2016) 1615-22. doi: 10.1093/infdis/jiw001
- [40] I. González, J. Gómez, M. Santander-Nelli, M. Natali, D. Cortés-Arriagada, P. Dreyse, Synthesis and photophysical characterization of novel Ir(III) complexes with a dipyrrophenazine analogue (ppdh) as ancillary ligand, *Polyhedron* 186 (2020) 114621. doi: 10.1016/j.poly.2020.114621
- [41] I. González, M. Natali, A.R. Cabrera, B. Loeb, J. Maze, P. Dreyse, Substituent influence in phenanthroline-derived ancillary ligands on the excited state nature of novel cationic Ir(III) complexes, *New Journal of Chemistry* 42(9) (2018) 6644-6654. doi: 10.1039/c8nj00334c
- [42] H. Ishida, S. Tobita, Y. Hasegawa, R. Katoh, K. Nozaki, Recent advances in instrumentation for absolute emission quantum yield measurements, *Coordination chemistry reviews* 254(21-22) (2010) 2449-2458. doi: 10.1016/j.ccr.2010.04.006
- [43] C.R. MacNair, J.M. Stokes, L.A. Carfrae, A.A. Fiebig-Comyn, B.K. Coombes, M.R. Mulvey, E.D. Brown, Overcoming mcr-1 mediated colistin resistance with colistin in combination with other antibiotics, *Nature communications* 9(1) (2018) 458. doi: 10.1038/s41467-018-02875-z
- [44] J.M. Stokes, C.R. MacNair, B. Ilyas, S. French, J.P. Cote, C. Bouwman, M.A. Farha, A.O. Sieron, C. Whitfield, B.K. Coombes, E.D. Brown, Pentamidine sensitizes Gram-negative pathogens to antibiotics and overcomes acquired colistin resistance, *Nature microbiology* 2 (2017) 17028. doi: 10.1038/nmicrobiol.2017.28

- [45] N.P. Weng, Aging of the immune system: how much can the adaptive immune system adapt?, *Immunity* 24(5) (2006) 495-9. doi: 10.1016/j.immuni.2006.05.001
- [46] M. Riou, L. Avrain, S. Carbonnelle, F. El Garch, J.P. Pirnay, D. De Vos, P. Plesiat, P.M. Tulkens, F. Van Bambeke, Increase of efflux-mediated resistance in *Pseudomonas aeruginosa* during antibiotic treatment in patients suffering from nosocomial pneumonia, *International journal of antimicrobial agents* 47(1) (2016) 77-83. doi: 10.1016/j.ijantimicag.2015.11.004
- [47] EUCAST, EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance, (2016) doi:
- [48] M. Brady, R. Cunney, S. Murchan, A. Oza, K. Burns, *Klebsiella pneumoniae* bloodstream infection, antimicrobial resistance and consumption trends in Ireland: 2008 to 2013, *European journal of clinical microbiology & infectious diseases* : official publication of the European Society of Clinical Microbiology 35(11) (2016) 1777-1785. doi: 10.1007/s10096-016-2727-4
- [49] C. Marques, J. Menezes, A. Belas, C. Aboim, P. Cavaco-Silva, G. Trigueiro, L. Telo Gama, C. Pomba, *Klebsiella pneumoniae* causing urinary tract infections in companion animals and humans: population structure, antimicrobial resistance and virulence genes, *The Journal of antimicrobial chemotherapy* (2018) doi: 10.1093/jac/dky499
- [50] T.A. Russo, R. Olson, U. MacDonald, J. Beanan, B.A. Davidson, Aerobactin, but not yersiniabactin, salmochelin, or enterobactin, enables the growth/survival of hypervirulent (hypermucoviscous) *Klebsiella pneumoniae* *ex vivo* and *in vivo*, *Infection and immunity* 83(8) (2015) 3325-33. doi: 10.1128/IAI.00430-15
- [51] J.M. Vargas, M.P. Moreno Mochi, J.M. Nunez, M. Caceres, S. Mochi, R. Del Campo Moreno, M.A. Jure, Virulence factors and clinical patterns of multiple-clone hypermucoviscous KPC-2 producing *K. pneumoniae*, *Heliyon* 5(6) (2019) e01829. doi: 10.1016/j.heliyon.2019.e01829
- [52] F.R. DeLeo, W.M. Nauseef, Granulocytic Phagocytes, in: D.R. Bennett JE, Blaser, MJ (Eds.), *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*, Elsevier, Philadelphia,, 2014, pp. 78–92.
- [53] M.A.J. Rodgers, Solvent-induced deactivation of singlet oxygen: additivity relationships in nonaromatic solvents, *Journal of the American Chemical Society* 105(20) (1983) 6201-6205. doi: 10.1021/ja00358a001
- [54] E. Wachter, D.K. Heidary, B.S. Howerton, S. Parkin, E.C. Glazer, Light-activated ruthenium complexes photobind DNA and are cytotoxic in the photodynamic therapy window, *Chemical communications* 48(77) (2012) 9649-9651. doi: 10.1039/C2CC33359G
- [55] L. Edwards, D. Turner, C. Champion, M. Khandelwal, K. Zingler, C. Stone, R.D. Rajapaksha, J. Yang, M.I. Ranasinghe, A. Kornienko, L.V. Frolova, S. Rogelj, Photoactivated 2,3-distyrylindoles kill multi-drug resistant bacteria, *Bioorganic & medicinal chemistry letters* 28(10) (2018) 1879-1886. doi: 10.1016/j.bmcl.2018.04.001
- [56] F. Li, J.G. Collins, F.R. Keene, Ruthenium complexes as antimicrobial agents, *Chemical Society reviews* 44(8) (2015) 2529-42. doi: 10.1039/c4cs00343h
- [57] I. González, P. Dreyse, Substituent influence in phenanthroline-derived ancillary ligands on the excited state nature of novel cationic Ir(III) complexes., *New J. Chem* 42 (2018) 6644-6654. doi:
- [58] A.C. Hachey, D. Havrylyuk, E.C. Glazer, Biological activities of polypyridyl-type ligands: implications for bioinorganic chemistry and light-activated metal complexes, *Current Opinion in Chemical Biology* 61 (2021) 191-202. doi: 10.1016/j.cbpa.2021.01.016
- [59] A. Wozniak, A. Rapacka-Zdonczyk, N.T. Mutters, M. Grinholc, Antimicrobials Are a Photodynamic Inactivation Adjuvant for the Eradication of Extensively Drug-Resistant *Acinetobacter baumannii*, *Frontiers in microbiology* 10 (2019) 229. doi: 10.3389/fmicb.2019.00229
- [60] D. Pletzer, S.C. Mansour, R.E.W. Hancock, Synergy between conventional antibiotics and anti-biofilm peptides in a murine, sub-cutaneous abscess model caused by recalcitrant ESKAPE pathogens, *PLoS pathogens* 14(6) (2018) e1007084. doi: 10.1371/journal.ppat.1007084